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Interaction of local anesthetics with lipid bilayers investigated by ^{1}H MAS NMR spectroscopy

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ABSTRACT

The membrane location of the local anesthetics (LA) lidocaine, dibucaine, tetracaine, and procaine hydrochloride as well as their influence on phospholipid bilayers were studied by ^{31}P and ^{1}H magic-angle spinning (MAS) NMR spectroscopy. The ^{31}P NMR spectra of the LA/lipid preparations confirmed that the overall bilayer structure of the membrane remained preserved. The relation between the molecular structure of the LAs and their membrane localization and orientation was investigated quantitatively using induced chemical shifts, nuclear Overhauser enhancement spectroscopy, and paramagnetic relaxation rates. All three methods revealed an average location of the aromatic rings of all LAs in the lipid-water interface of the membrane, with small differences between the individual LAs depending on their molecular properties. While lidocaine is placed in the upper chain/glycerol region of the membrane, for dibucaine and procaine the maximum of the distribution are slightly shifted into the glycerol region. Finally for tetracaine the aromatic ring is placed closest to the aqueous phase in the glycerol/headgroup region of the membrane. The hydrophobic side chains of the LA molecules dibucaine and tetracaine were located deeper in the membrane and showed an orientation towards the hydrocarbon core. In contrast the side chains of lidocaine and procaine are oriented towards the aqueous phase.

1. Introduction

Local anesthetics (LA) are well-known pharmaceutical agents consisting of a lipophilic aromatic ring structure and at least one side chain both connected by an intermediate carboxyl group, generally either an ester or an amide bond. The chemical structure and the physicochemical properties of LAs determine their biological activity [1]. The local anesthetic potency is highly dependent on the length of the side chain, and the LA specific physicochemical properties are determined by the nature of the substituent at the aromatic residue or at the amino group [2]. Furthermore, the respective chemical structure determines where and how the metabolism takes place as well as the spectrum of systemic and local side effects of the LA. In addition, the potency as well as the toxicity of the LAs are directly related to the hydrophobicity of the LA molecules [3–6].

Although the mode of action of LA is not yet fully understood, it is currently considered that they are binding to voltage-gated Na^{+} channels, thus blocking nerve impulses by blocking the action potential of axons [1,7–10]. Many LAs are ionizable amines and both protonated and neutral LA are known to be able to bind to these channels while the neutral LAs bind stronger to the nerve membranes than the protonated forms [1,9,12,13]. The detailed molecular mechanism of interrupting nerve impulses by the LAs remains still unclear but the lipid membrane binding and the location of the LAs within the phospholipid membrane can provide useful information for the understanding of the mode of action of the LAs [11]. Membrane binding restricts the diffusion of the LA molecules to two dimensions, which dramatically enhances the probability of interactions with the membrane channels. Nevertheless, an incorporation of the LA deeply into the hydrocarbon core of the phospholipid membrane may also prevent the LA from binding to the voltage-gated Na^{+} channels, if the binding site on the channel is situated in the membrane-water-interface.

It is known that general and local anesthetics modify the physicochemical properties of lipid bilayers and that LAs increase the rotational mobility of membrane lipids [14]. Furthermore, Fraceto et al. [5] have assumed that the preferential orientation and positioning of LAs inside the lipid membrane is determined by its hydrophobicity, sterical hindrances and uncharged/charged ratio at pH 7.4 [6,15,16]. In addition a limited penetration of LAs into the hydrophilic headgroup region of lipid bilayers was proposed and an increase of the electrical conductance of phospholipid bilayers in the presence of LAs was observed [17–19]. Previous studies also showed that the polar moiety of LA interacts with polar phospholipid segments and the lipophilic moiety of the LA inserts into the hydrophobic region of the lipid bilayer [9,20–22].

Abbreviations: LA, local anesthetics; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 5/10/16-doxyl-PC, 1-palmitoyl-2-stearoyl-(5/10/16-doxyl)-*sn*-glycero-3-phosphocholine; tempo-PC, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(tempo)choline; DMPC-d_{67}, 1,2-dimyristoyl(d_{54})-*sn*-glycero-3-phosphocholine-1,1,2,2-d_4-N,N,N-trimethyl-d_9; MAS, magic angle spinning; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy

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In spite of many studies, it is not yet fully known which phospholipid bilayer regions interact with LAs or what membrane structure modification is responsible for the induction of the LA effects [18,23–25] and the exact way of binding of LA to the phospholipids is still discussed controversially [11,16,26,27].

In the past, the interaction of LAs with lipid bilayers has been studied by a number of techniques including ^{1}H NMR [5,28–32], ^{2}H NMR spectroscopy [9,20,28,31–34], UV-spectroscopy [35], fluorescence experiments [4,36], atomic force microscopy [37], capillary electrophoresis [38] and differential scanning calorimetry [22,24,39]. In summary, all these studies showed that LAs have different effects on the order, organization, mobility and general properties of phospholipid bilayers. Furthermore, it was noted that the preferential location and orientation of the LAs inside the bilayer is determined by their hydrophobic and steric properties and the fact that the various LAs are located in different depths of the phospholipid bilayer. Additionally, it was found out that the charged and uncharged forms of the LAs exhibit different locations inside the bilayer indicating a pH dependence of the binding of the LAs to the membrane. In these studies a lot of important information were gained, but most of the methods can only report a rough membrane position and no orientation of the molecule in the membrane. Sometimes the proposed membrane models are based only on qualitative data analysis.

In this study the location of four different LAs and their influence on lipid bilayers as a result of the interaction between phospholipid molecules and the LAs were studied by several NMR techniques. NMR spectroscopy is a powerful method to study the molecular order, structure and dynamics of lipid bilayers and the molecules bound to them [40–42]. For the first time we use here a quantitative analysis of ^{1}H nuclear Overhauser effect (NOE) spectroscopy (NOESY) data to obtain the membrane location and orientation of LAs in a phospholipid membrane, a method that was successfully used for a number of small molecules in lipid membranes [41,43,44]. With this quantitative analysis of the ^{1}H MAS NOESY NMR cross-relaxation rates between protons of the LA and membrane phospholipids, additionally confirmed by the quantitative measurement of the paramagnetic relaxation rates using spin labels located in different membrane depths, a distribution profile of the LA with respect to the lipid bilayer could be obtained. Further, a preferred orientation of the LA molecules in the membrane could be achieved by comparing the membrane distribution profiles of different molecular groups of one LA. These quantitative information leads to advanced and very precise model of the membrane position and orientation of the investigated LA with in lipid membrane, which gives more detailed insights into the first step in the mode of action of the investigated LAs.

Four different LAs with different putative membrane properties for instance their hydrophobicity were chosen to obtain a broad overview on the membrane interaction of the LAs. With the sometimes small structural differences between the molecules, insights how these differences alter the LA-membrane interaction and therefore the membrane position and orientation of the LA are obtained. This can also provide hints how the structural differences leads to differences in the action of LA. Therefore, we used lidocaine, dibucaine, tetracaine, and procaine hydrochloride (Fig. 1). Lidocaine is worldwide the most commonly used and best studied fast acting LA molecule.

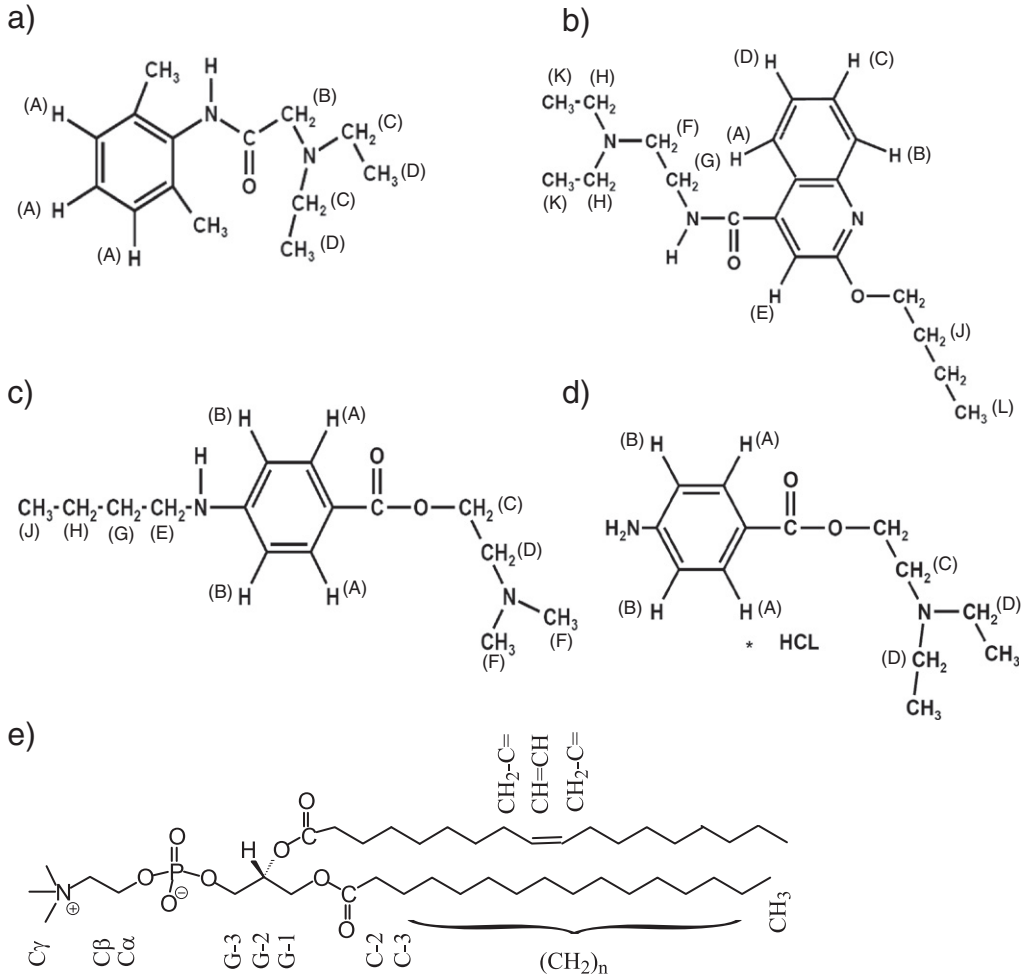

Fig. 1. Chemical structures of a) lidocaine, b) dibucaine, c) tetracaine, d) procaine hydrochloride and e) POPC. The capital letters refer to the respective peaks assigned in the ^{1}H MAS NMR spectra shown in Fig. 2.

It belongs to the amide family and possess only one short side chain. Lidocaine is present in both the charged and the uncharged forms [8]. Dibucaine, also known as cinchocaine, is a tertiary amino amide local anesthetic agent existing both in neutral and cationic forms at physiological pH [45]. Dibucaine is a much bigger molecule than lidocaine and consist of two fused aromatic rings and two sides chains, where at least one is clearly hydrophobic as a pure fatty acid chain. Tetracaine belongs to the ester group of the LAs and is a long-acting agent at a low therapeutic dose [46]. Tetracaine possesses also two side chains similar to dibucaine. Procaine is the oldest synthetic and injectable local anesthetic. It is used as an injection and nerve block anesthesia because of its slow onset and short period of effect [47]. Procaine has a quite similar molecular structure like lidocaine but is an ester type LA.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-stearoyl-(5/10/16-doxyl)-*sn*-glycero-3-phosphocholine (5/10/16-doxyl-PC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(tempo)choline (tempo-PC) and 1,2-dimyristoyl(d_{54})-*sn*-glycero-3-phosphocholine-1,1,2,2-d_4-N,N,N-trimethyl-d_9 (DMPC-d_{67}) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and LA – lidocaine, dibucaine, tetracaine, and procaine hydrochloride – were purchased from Sigma–Aldrich Chemie GmbH (Munich, Germany). All reagents were used without further purification. The chemical structures of the LA and POPC are shown in Fig. 1.

2.2. Sample preparation

For the ^{31}P and ^{1}H MAS NMR measurements, mixtures of POPC and the LA (20 mol%) were dissolved in chloroform (lidocaine, dibucaine, tetracaine) or in a chloroform/methanol mixture (procaine hydrochloride). For the measurements of the paramagnetic relaxation rates POPC was replaced by DMPC-d_{67} and 0.5 mol% spin-labeled phospholipids (5/10/16-doxyl-PC or tempo-PC) were added. After evaporating the organic solvent under vacuum, the samples were re-dissolved in cyclohexane and lyophilized at high vacuum to obtain a fluffy powder. After hydration with 40 wt.% D_2O buffer (10 mM NaCl, 50 mM HEPES, pD 7.4) the samples were equilibrated by freeze–thaw cycles and gentle centrifugation and finally transferred into 4 mm HR MAS rotors.

2.3. Solid-state NMR spectroscopy

All NMR measurements were carried out on DRX-600 or Avance III 600 NMR spectrometers (Bruker Biospin GmbH; Rheinstetten, Germany) at a temperature of 303 K.

For the ^{31}P NMR spectra, the system was operating at a resonance frequency of 242.8 MHz. A Hahn-echo pulse sequence with a typical 90° pulse length of 10 μs, an echo delay of 50 μs, a spectral width of 100 kHz and a relaxation delay of 2.5 s were used. Low-power broadband ^{1}H decoupling was applied during acquisition. To obtain the chemical shift anisotropy ($\Delta\sigma$) the resulting spectra were simulated using Mathcad 14.0 M020 (Parametric Technology Corporation, Needham, MA, USA).

For the ^{1}H MAS NMR measurements a 4 mm HR MAS probe was used. The ^{1}H MAS NMR spectra were carried out at a spinning frequency of 8 kHz. Typical $\pi/2$ pulse lengths were around 10 μs. A ^{2}H lock was used for field stability. The ^{1}H NMR spectra were referenced with respect to the terminal methyl group of the lipid chains at 0.885 ppm for a POPC sample in absence of LA molecules [48]. The assignments of the specific LA ^{1}H signals were determined according to the Spectral Database for Organic Compounds SDBS (http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/cre_index.cgi?lang=eng) for lidocaine, tetracaine, and procaine and obtained from literature (lidocaine [4,5]; dibucaine, tetracaine and procaine [30]). One should note the ^{1}H MAS NMR spectra in these papers were calibrated to 0 ppm for the terminal methyl group of the phospholipid acyl chains and not to 0.885 ppm as it is correct [48].

Two-dimensional ^{1}H MAS NOESY spectra were acquired at five mixing times (1, 100, 200, 300 and 500 ms). Typically, between 548 and 750 data points were acquired in the indirect dimension with 16 scans per increment at a relaxation delay of 3.5 s. The volumes of the respective diagonal and cross peaks were integrated using the Bruker Topspin 2.1 software package. Cross-relaxation rates were obtained from fitting the experimental cross peak volumes at varying mixing times using the nonlinear regression curve fitter in Origin 6.1 (OriginLab Cooperation, Northampton, MA, USA) according to the spin-pair-model described in literature [41,49].

T_1 relaxation times were measured using the inversion recovery pulse sequence with 9 delays between 1 ms and 5 s and a relaxation delay of 5 s. A fast paramagnetic relaxation mechanism is introduced by the unpaired electron of the spin label, therefore, the total relaxation rate $R_1 = 1/T_1$ is the sum of paramagnetic ($R_{1,p}$) and dipolar relaxation rates ($R_{1,d}$). After measuring $R_{1,d}$ from a sample without a spin labeled phospholipid, $R_{1,p}$ can be easily determined for each observable signal of the LA from the total relaxation rate R_1. It was shown that the doxyl labels bound phospholipid molecules exhibit an averaged membrane position according to their position in the phospholipid molecule [42].

3. Results and discussion

3.1. Comparison of the membrane interaction of the LAs

To compare the membrane interaction of the four different LAs static ^{31}P NMR und ^{1}H MAS NMR measurements were used.

At first to confirm that the phospholipid membranes were in the biologically relevant liquid crystalline phase state and to study the influence of membrane binding of the LAs to the lipid headgroups ^{31}P NMR spectra (Supplementary Fig. S1) were acquired. For all samples, the typical powder pattern line shapes of phospholipid membranes in the liquid crystalline phase were obtained, for some samples a small isotropic contribution of 1–2% of the spectral intensity is visible, which originates from small and highly curved structures. While for dibucaine ($\Delta\sigma = 46.8$ ppm) and tetracaine ($\Delta\sigma = 47.8$ ppm) the chemical shift anisotropy remained nearly unchanged compared to pure POPC membranes ($\Delta\sigma = 47.0$ ppm), this value decreased to $\Delta\sigma = 44.3$ ppm in the presence of lidocaine and increased to $\Delta\sigma = 52.0$ ppm in the presence of procaine hydrochloride. Such changes of the ^{31}P chemical shift anisotropy are caused by changes in the amplitude of molecular wobble (headgroup mobility) or in the orientation of the phospholipid headgroups. Obviously the incorporation of lidocaine and procaine leads to changes in these properties.

Deeper insights into the membrane interaction and mainly the position within the phospholipid membrane can be achieved by ^{1}H MAS NMR measurements of POPC membranes in the presence of the different LAs. The ^{1}H MAS NMR spectra (Fig. 2) exhibit well resolved phospholipid and LA signals.

Due to the ring current effect of the delocalized π–electron system of the aromatic rings in the LA molecules the POPC signals were slightly shifted to higher magnetic fields [51]. As a result of the high mobility and molecular disorder in lipid membranes, all POPC signals were affected, but the magnitude of these shifts depended on the ring orientation, the distance to the aromatic ring and the probability of the interaction between the aromatic ring of the LAs and the molecular segments of POPC [43]. Therefore, the analysis of induced chemical shift values offers a simple way of obtaining an average location of the aromatic ring of the different LAs in the phospholipid membrane by plotting these values for each phospholipid segment along the long

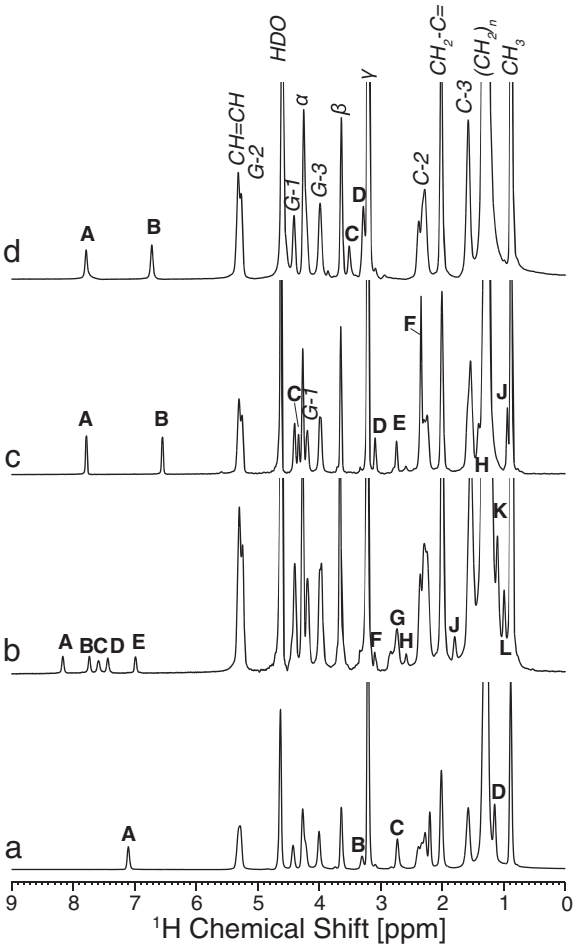

Fig. 2. 600.1 MHz ^{1}H MAS NMR spectra of POPC multilamellar vesicles in the presence of 20 mol% of a) lidocaine, b) dibucaine, c) tetracaine and d) procaine hydrochloride. The measurements were carried out at a D_2O buffer content of 40 wt.%, a temperature of 303 K and a MAS spinning frequency of 8 kHz. The assignments of the local anesthetic peaks (bold) refer to the respective protons marked in Fig. 1 a–d. The assignment of the POPC signals is given in spectrum d (italic).

axis of the molecule as shown in Fig. 3. For all four LAs a broad distribution function was obtained, which is caused by the high molecular mobility and disorder in phospholipid membranes. Dibucaine and procaine hydrochloride exhibited a clear maximum in the distribution function, which corresponds to the average membrane location of the aromatic ring of these molecules in the upper chains and headgroup region, respectively. For lidocaine a maximum was difficult to determine, there could be a tendency for a preferred localization in the upper chain or the headgroup region. For tetracaine no clear maximum could be observed.

The quantitative determinations of NOESY cross-relaxation provide a closer insight into the membrane location of the four different LAs in phospholipid membranes. For the investigated LAs, especially the protons bound to the aromatic rings gave clearly resolved cross peaks in the NOESY NMR spectra (not shown), which could be analyzed quantitatively. As observed for other molecules before [41,43,52], these protons exhibited interactions with all phospholipid segments from the headgroups to the acyl chain ends (Fig. 4) due to the high molecular disorder and dynamics in phospholipid membranes. Since the cross-relaxation rate is strongly distance depended, these plots can be interpreted as distribution function of the respective molecular group along the membrane normal. The maximum of the obtained distribution functions is relatively well visible in the lipid-water interface region for the aromatic ring of all four LAs, as already suggested from the induced chemical shifts. None of the LAs was buried deeply in the hydrocarbon core of the membrane. Such a preferred location is caused by the physical interactions in the lipid membrane interface. Since all molecules carry polar and nonpolar molecular segments, the whole complex interplay of interactions determines the membrane position of a molecule in the membrane [41,53]. In addition, for the aromatic rings the cation-π-interaction [54] places these rings in the membrane-water-interface, as it was also observed for aromatic amino acids [55].

Nevertheless some interesting differences in the membrane distribution profiles of the four LAs as shown in Fig. 4 can be seen. For lidocaine the aromatic ring seems to have the deepest average position in the phospholipid membrane since here a maximum of the distribution function was found for upper chain region (around C-3). For dibucaine and procaine hydrochloride the average position is shifted somewhat upwards with a maximum of the distribution function in the glycerol region of the membrane. For tetracaine the aromatic ring was located in the glycerol/headgroup region of the membrane and therefore closes to the aqueous phase. The small differences have their origin in the very complex physical interaction pattern between the molecules in a phospholipid membrane, which can be change already by even small differences in the molecular structures.

3.2. Membrane orientation of the different investigated LAs

3.2.1. Lidocaine

The analysis of the NOESY data also allows concluding the orientation of the LAs in the membrane by comparing the distribution profiles for different protons of one LA molecule. In Fig. 5, this is shown for the example lidocaine, which exhibits the deepest membrane position of the investigated LAs for its aromatic ring as seen above. All well resolved protons of lidocaine (A, B, and C) exhibit again broad distribution functions with maxima in the upper chain/glycerol region of the POPC membrane. The differences between these distribution functions are rather small. For the protons D of lidocaine a quantitative analysis of the cross-relaxation rates using the spin-pair-model was unfortunately not possible due to signal overlap or the absence of clearly visible cross peaks for short mixing times. The observation of cross peaks between the D protons of lidocaine and the molecular groups $CH_2-C=$ and C-3 of POPC for longer mixing times lead nevertheless at least to qualitative information on the average location of these protons.

These results can be confirmed by measurements of the paramagnetic relaxation rates induced by phospholipids carrying a paramagnetic spin label group in different depths of the membrane. Since these paramagnetic relaxation rates also exhibit a strong distance dependence and the paramagnetic labels exhibit a membrane position according their binding place in the phospholipid molecule, spin labels in different membrane positions can be used to determine the average membrane location of a respective molecular group [42,50,56] and here especially the orientation of the side chains of the LAs. By the interpretation of these measurements one should keep in mind that the paramagnetic labels are also subject to the high molecular disorder and mobility in lipid membranes [42]. The paramagnetic relaxation rates for the protons A, B and C of lidocaine are shown in Fig. 6. Note that protons D of lidocaine could not be analyzed because of signal overlap with DMPC-d_{67}. The largest paramagnetic relaxation rates for all three molecular groups of lidocaine were observed for the headgroup spin labeled tempo-PC. While the paramagnetic relaxation rates decreases if the doxyl-label is shifted downwards the acyl chain of the phospholipid for the protons B and C of the side chain of lidocaine. For the protons A on the aromatic ring in the presence of 5- or 10-doxyl PC similar paramagnetic relaxation rates were measured. Therefore, one has to assume a position of lidocaine between the upper acyl chain and the headgroup region of the phospholipid membrane and the side chain of the molecule is oriented towards the aqueous phase. Also, a fast rotation of such a small

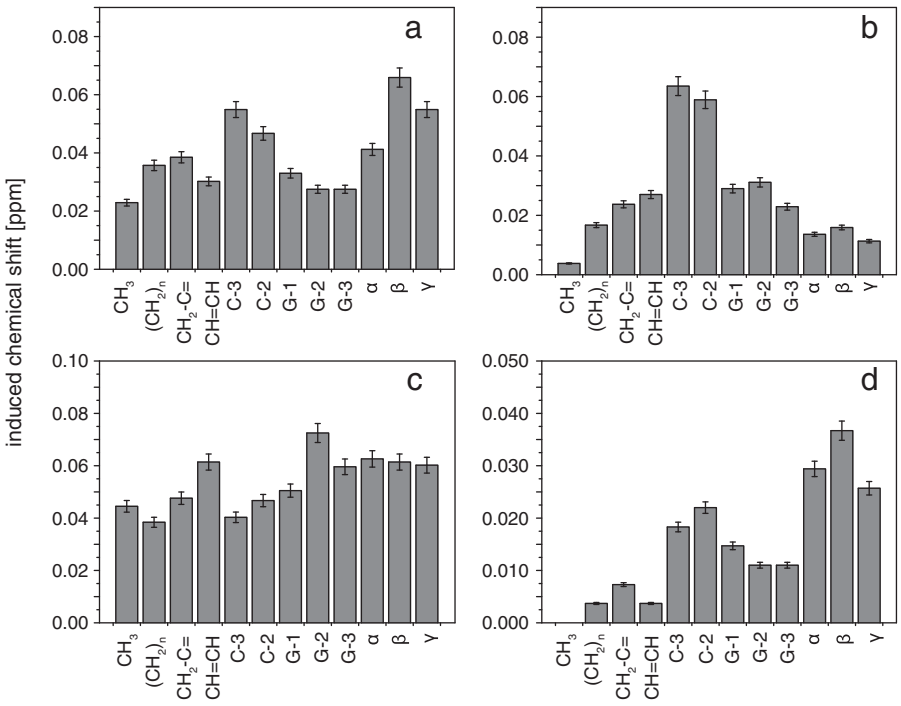

Fig. 3. Induced chemical shift changes (ppm) of the POPC lipid signals in the presence of 20 mol% a) lidocaine, b) dibucaine, c) tetracaine and d) procaine hydrochloride.

molecule in the membrane seems possible. Such a membrane location is consistent with earlier measurements for lidocaine by small-angle X-ray scattering and fluorescence experiments, which found that lidocaine hydrochloride is oriented in the lipid interface region and the ionic part of the molecule is exposed to water [57]. For charged lidocaine, a stronger interaction with the lipid headgroups [22] and an influence of lidocaine on the membrane organization was found [4,9], which is also reflected in our ^{31}P NMR data. Additionally, it was observed that only the uncharged form of LA can cross the membrane while this is not possible for the charged form [8]. Under our experimental conditions both the charged and uncharged form of lidocaine should be present with a slight predominance of the protonated form [1,25,35,57]. These differences in the membrane properties between the charged and the uncharged form of lidocaine can

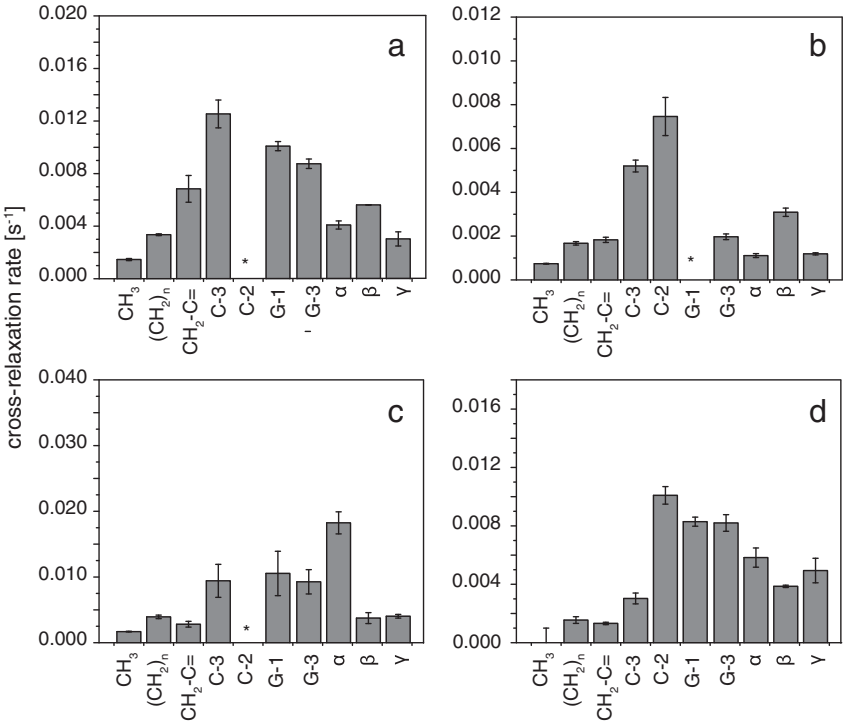

Fig. 4. Comparison of the cross-relaxation rates (s^{-1}) between protons of the aromatic ring of the different local anesthetics and POPC lipid segments (a) lidocaine protons A, b) dibucaine proton C, c) tetracaine protons A and d) procaine hydrochloride protons B as assigned in Fig. 1 a–d). * Cross-peaks were not analyzed because of peak overlap between LA and POPC signals.

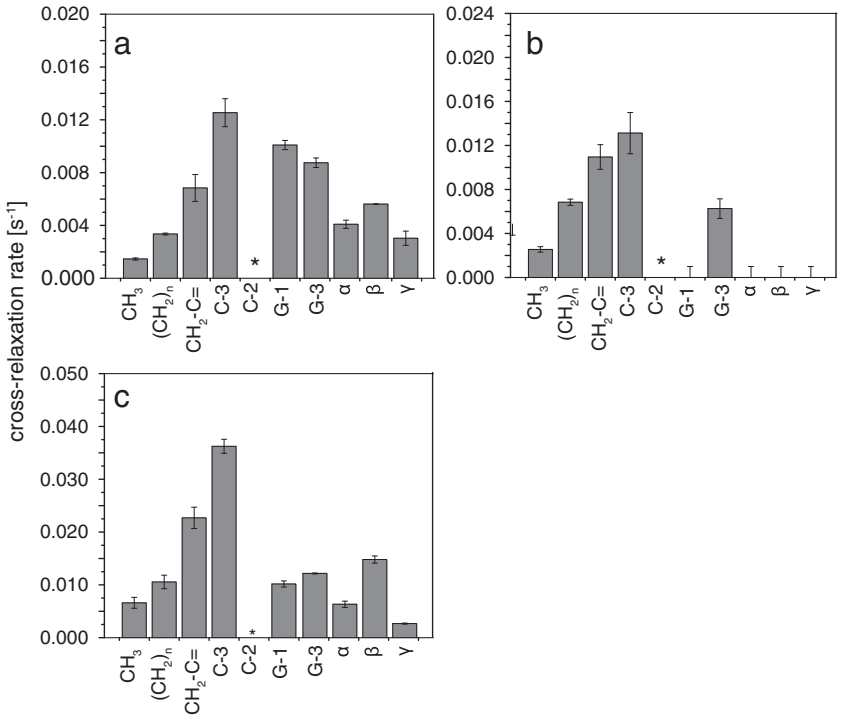

Fig. 5. Cross-relaxation rates (s^{-1}) between the protons of lidocaine and the lipid segments of POPC at a concentration of 20 mol%. A, B and C refer to the respective protons assigned in Fig. 1 a. * Cross-peaks were not analyzed because of peak overlap between lidocaine and POPC signals.

be explained by increased electrostatic interactions and the Born repulsion, which prevent the charged form from a deeper insertion into the lipid membrane.

3.2.2. Dibucaine

For second investigated LA dibucaine, for which the aromatic ring is placed in the glycerol region of the membrane (see above), cross-relaxation rates to lipid segments of POPC could be determined for the protons A, B, C, D and E of the quinoline ring (see Supplementary Fig. S2). Again, broad distribution functions with maxima in the membrane interface were achieved. The maxima are found in the upper chain (C-2) or headgroup region depending on the proton investigated. Since for the protons A, D and E the distribution functions seems to be shifted towards the lipid headgroup region an orientation of the quinoline ring with these protons towards the aqueous phase can be assumed. In addition, resolved cross peaks for the proton G, J, K and L were observed qualitatively. For the hydrophobic acyl chain with the ether bond, the protons J and L exhibit cross peaks to $CH_2-C=$ and C-3 or $(CH_2)_n$ and C-3 of POPC, respectively. Therefore, this chain seems oriented directly downwards into the hydrocarbon core of the membrane. For the other side chain of dibucaine, the proton G shows cross peaks to the molecular groups γ and β of the POPC molecule, while the proton K exhibit cross peaks to $(CH_2)_n$ and C-3. So this side chain seems in agreement

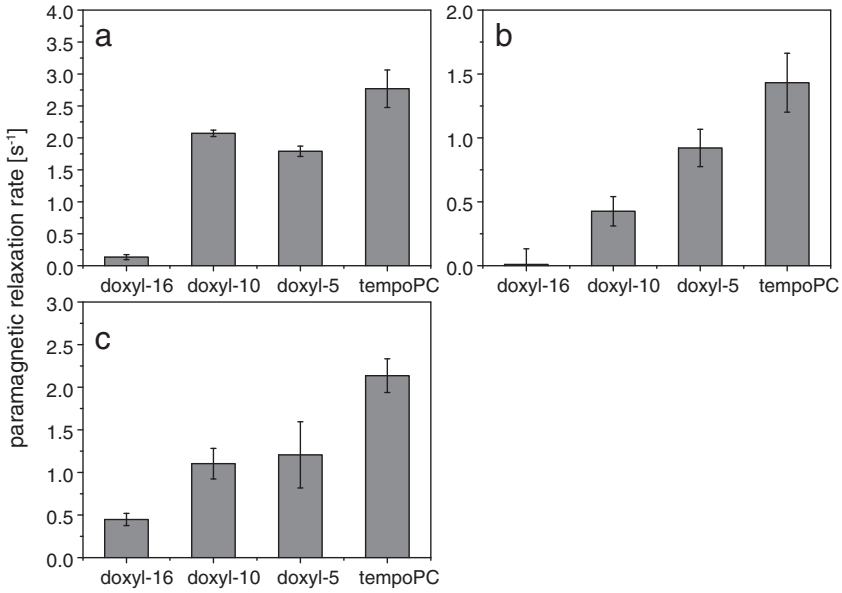

Fig. 6. Paramagnetic relaxation rates (s^{-1}) for the protons of lidocaine in the presence of 5-, 10-, 16-doxyl-PC, or tempo-PC at a molar ratio of 0.5 mol%. A, B and C refer to the respective protons assigned in Fig. 1 a.

with the orientation of the quinoline ring (see above) to undergo a turn which places proton G into the headgroup region and the chain end protons K into upper chain region of the phospholipid membrane. Due to the general location of the molecule in the glycerol region, the short length of the dibucaine side chain (compared to the POPC acyl chain) and the high mobility in the membrane, also the cross peaks of the side chain protons of dibucaine to the lipid headgroup can be explained. These results correspond well to the data published by Kuroda et al. [31]. In addition, the work of Lorite et al. [37] revealed by atomic force microscopy, adsorption kinetics and dilatational elasticity measurements also the dibucaine interaction with the polar headgroups of lipids thus causing a modification of the molecular packing of phospholipid bilayers. This behavior of dibucaine can be explained by its molecular properties — the quinoline ring together with the carbonyl group are the more polar parts of the molecule while especially the acyl chain is rather hydrophobic and have, therefore, to be inserted into the phospholipid membrane. The paramagnetic relaxation data seems a little bit contradictory to these results. For all protons, for which an analysis of the paramagnetic relaxation rates was possible, a maximum for 10-doxyl was found (Suppl. Fig. S3). This would lead to a deeper membrane location of the whole molecule as stated above based on the NOESY results. A possible explanation could be that dibucaine forms very unstable face-to-face π-stacked dimers in phospholipid membranes [29,31,58]. A breakup of such dimers in the membrane may lead to different results for the membrane location in our various measurements.

3.2.3. Tetracaine

For tetracaine, where the aromatic ring is places quite close to the aqueous phase as shown above, cross-relaxation rates to the POPC signals were determined for the protons A, B and C (Suppl. Fig. S4). The protons A and B bound to the aromatic ring exhibited similar cross-relaxation rate profiles with a maximum in the glycerol/upper chain region of POPC. The paramagnetic relaxation rates for these protons (Suppl. Fig. S5) exhibited no clear trend for their location as it was also observed from the induced chemical shifts for tetracaine. Nevertheless, an average location of the aromatic ring of tetracaine in the glycerol region of the membrane seems probable. The proton C of ester bound side chain of tetracaine exhibited also a distribution function with a maximum in the glycerol region (Suppl. Fig. S4). Further, for this side chain cross peaks (again a quantitative analysis was not possible due to peak overlap) between the proton D of tetracaine and the molecular groups G-1 and G-3 of the POPC molecule, as well as between the protons F and the molecular groups $(CH_2)_n$, C-3 and C-2 were observed. Also, in the paramagnetic relaxations rates there was a tendency that the protons F exhibit stronger interactions with the acyl chains than the proton D (Suppl. Fig. S5). Therefore, one can assume that the end of this side chain points downwards towards the hydrophobic core of the membrane. For the second amide bound chain of tetracaine, NOESY cross peaks could be observed qualitatively between the proton E and the molecular group G1 of POPC, between the proton H and the molecular groups $(CH_2)_n$ and C-3, and finally between the proton J of tetracaine and the molecular groups CH_3 and $(CH_2)_n$. Also the paramagnetic relaxation rates for the tetracaine protons (Suppl. Fig. S5) argue in favor of an interaction of these protons with the acyl chains of the phospholipids. So one has to conclude, that the amide bound side chain of tetracaine is inserted into the chain region of phospholipid membrane due do its hydrophobic nature. While the polar part of the tetracaine molecule (aromatic ring with the bound NH and C═O group) is located in the glycerol region, both chains (and especially the pure acyl chain) are oriented towards the hydrocarbon core of the membrane. These results can be underlined by earlier studies, which report that tetracaine molecules interact with the headgroup region of phospholipids and have a disordering effect on the acyl chains as reported in earlier ^{31}P NMR,

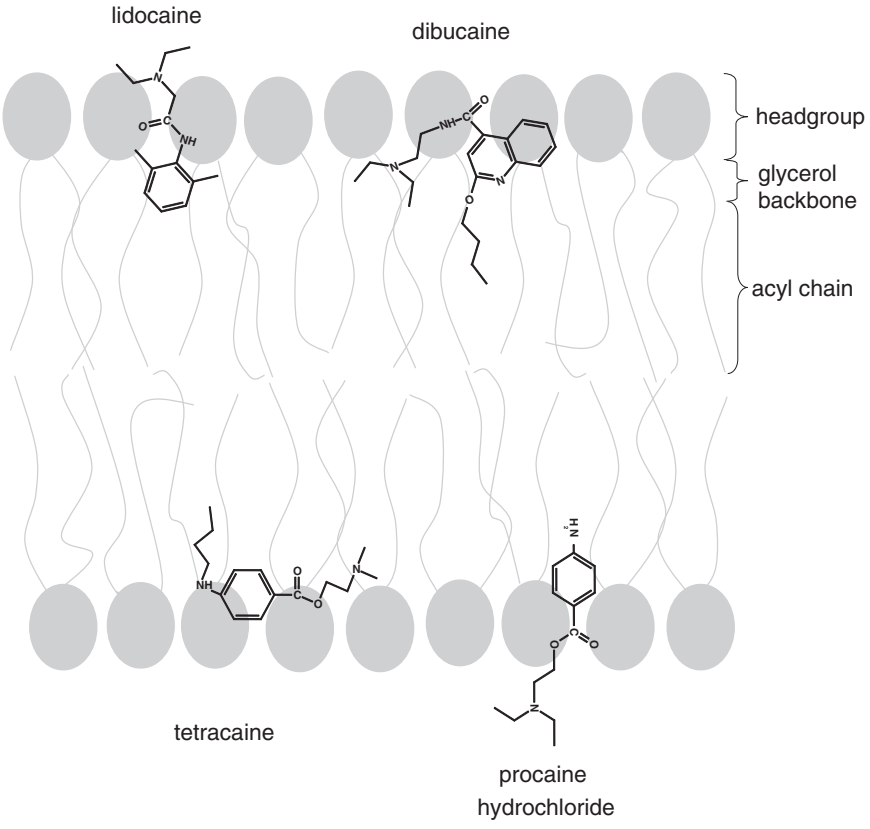

Fig. 7. Cartoon illustrating the average membrane location and orientation of the LAs lidocaine, dibucaine, tetracaine and procaine hydrochloride in a POPC membrane.

^{1}H and ^{2}H NMR and fluorescence studies [20,27,30,32,59]. Interestingly, using ^{2}H NMR Boulanger et al. [20] and Kelusky et al. [26] found that charged and uncharged tetracaine occupy different sites in phospholipid bilayers indicated by a strong pH dependence of the quadrupole splittings. Uncharged tetracaine – as used in this study – is primarily interacting with the hydrocarbon region.

3.2.4. Procaine

Finally, procaine hydrochloride is a LA with a molecular structure quite similar to lidocaine, except procaine hydrochloride is an ester type LA (in contrast to lidocaine being an amide type) and one NH_2 group of procaine hydrochloride in contrast to the two CH_3 groups bound to the aromatic ring of lidocaine. Compared to lidocaine, procaine exhibits an average membrane position which is slightly shifted into the glycerol region and similar to dibucaine.

For procaine hydrochloride a quantitative analysis of the cross-relaxation rates was only possible for the protons of the aromatic ring (A and B) (Suppl. Fig. S6). Both distribution functions exhibit a maximum in the glycerol region of the POPC membrane. This means that the aromatic ring of procaine hydrochloride is slightly shifted upwards in comparison to lidocaine, as already observed from the induced chemical shift data. Obviously already the slightly increased hydrophobicity due to the additional two CH_3 groups of lidocaine causes this small difference. This difference can be also explained by the higher lipid solubility of lidocaine (366) in comparison to procaine (100), which is defined as a partition coefficient between an organic solvent (e.g. octanol) and an aqueous buffer medium and determines in general the relative anesthetic potency [60]. The relationship between lipid solubility and anesthetic potency ensures that higher lipophilic LAs penetrate the nerve membranes faster than less lipophilic LAs [60]. In contrast to lidocaine, no intermolecular cross peak to POPC for side chain protons of procaine (C and D) could be observed. This result leads to the assumption that the side chain of procaine is oriented towards the aqueous phase similar to lidocaine, but may further be placed into the aqueous phase than for lidocaine. This assumption can be confirmed by the paramagnetic relaxation rates (Suppl. Fig. S7), while for the protons A and B of the aromatic ring no clear trend is observable, the protons D exhibit a stronger interaction with 5-doxyl and tempo-PC compared to the doxyl-labels that reside deeper in the hydrophobic core of the membrane. This orientation of the procaine molecule was suggested before by ^{2}H NMR measurements in phosphatidylethanolamine membranes [34]. This location of the whole molecule in the headgroup region of the membrane also explains the influence of procaine hydrochloride on the ^{31}P NMR spectra and therefore the mobility or orientation of the phospholipid headgroups, as discussed above. Previous studies also demonstrated that procaine exhibit only weakly interactions with phospholipid bilayers at the outer surface of the vesicles [11,30,47].

4. Conclusions

In the current study, we have carried out quantitative ^{1}H MAS NMR measurements to determine the location and orientation of the local anesthetics lidocaine, dibucaine, tetracaine, and procaine hydrochloride in phospholipid bilayers. Our results showed that the aromatic ring structures of all analyzed LA are localized in the lipid-water interface of the membrane defined by the upper chain/glycerol/headgroup region. A cartoon illustrating the average position and orientation of the LA molecules in a phospholipid membrane according to our results is given in Fig. 7.

The side chains of lidocaine and procaine are mainly oriented towards the aqueous phase, whereas at least the hydrophobic side chains of the LA molecules dibucaine and tetracaine point into the hydrocarbon core of the membrane. In the highly dynamic and disordered membranes with their complex interplay on very different physical interactions already small changes in molecular structures can modify the membrane location of incorporated molecules. Of course we can only speculate about the further interaction of the LAs with the Na^+ channels as a second step in their mode of action. The location in the membrane-water interface enables the LAs to bind to the voltage-gated Na^+ channels and may block the channel directly at the entrance. This would be probably prevented if the LAs were deeply buried in the hydrocarbon core of the cell membrane. The other possibility of a binding of the LA to the channel deep inside the lipid membrane, which could cause structural changes in the channel seems not very likely from the determined membrane locations. The investigation of interaction of the LAs after the membrane binding with the voltage-gated Na^+ channels remains a challenging task for further studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.07.014.

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